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Oncogene RNA Protein Binding and Breast Cancer  
Invasion

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## Introduction

In breast cancer, the propensity of breast cancer cells to invade and metastasize to lymph nodes, is the primary determinant of poor outcome. This year, we have made significant progress toward our goal of elucidating the mechanism by which the *c-fms* proto-oncogene contributes to aggressive invasive behavior of breast cancer cells. We have discovered that there is likely a complex of glucocorticoid simulated proteins which bind the 3' untranslated region of the *c-fms* RNA, and are well on our way to cloning some of the major proteins. Further, we have used microarray analysis to identify five genes overexpressed by dexamethasone in breast cancer cells. We have shown that one of them, *sgk*, appears to be integral to stimulation of *c-fms* related adhesiveness of breast cancer cells to extracellular matrix. Interference of phosphorylation of *sgk* significantly decreases both dexamethasone and CSF-1 (the *c-fms* ligand) induced adhesiveness.

## Body

The following details the progress made on each of the tasks outlined in the approved Statement Of Work.

### **Task 1. To clone the novel glucocorticoid (GC) stimulated *c-fms* RNA binding protein.**

#### **a. Biochemical protein purification followed by oligoRNA affinity chromatography (months 1-10)**

We used Northwestern analysis (Sela-Brown, A., Silver, J., Brewer, G., and Neveh-Many, T.J. Biol. Chem. 275: 7424-7429, 2000) to identify proteins which bind the 3' UTR of *c-fms* mRNA. One hundred micrograms of nuclear protein extract from BT20 breast cancer cells which had been treated for 16 hours with dexamethasone were separated by size on a 10 % SDS-PAGE gel. Proteins were transferred to a membrane and reacted with labeled *c-fms* 3' UTR or *c-fms* 3'UTR RNA from which 69nt had been deleted. Our preliminary results contained in the original submission had suggested that the *c-fms* RNA binding site may be contained within these 69 nt. We found that eight protein bands bound the *c-fms* RNA while only two bound the deletion RNA (Figure 1.). The 37 and 25 KDa bands bound both probes, while the 125, 100, 75, 70, 68 and 50KDa bands bound only the *c-fms* wild type 3' UTR RNA.

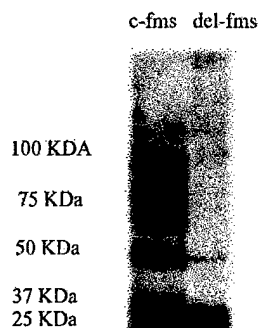


Figure 1. Northwestern Analysis of Nuclear Protein Extracts. 100ug nuclear protein extracts isolated from dexamethasone treated cells were size fractionated on 10% SDS-PAGE gels. Proteins were transferred to a PVD membrane by electrophoresis. The membrane was blocked for 1hour at RT in binding buffer (5mM Hepes (pH 7.4), 25mM KCl, 1.25mM MgCl<sub>2</sub>, 1.5mM

ATP, 0.1mM DTT) containing 0.5% BSA. The membrane was washed 2X in binding buffer and then was reacted with  $P^{32}$  labeled 3' c-fms or del-fms UTR RNA for 30 minutes. The membrane was washed 3X for 10 minutes at RT with binding buffer and then autoradiographed for 16 hours at  $-80^{\circ}\text{C}$ . Migration of the protein size markers is indicated.

This data indicates that there may be a complex of dexamethasone stimulated proteins which bind the 3'UTR of c-fms RNA. Further, some of these may bind to the 69nt fragment identified in the preliminary results, and others may bind to the 3' UTR of c-fms RNA outside of this 69nt region.

Using the purification scheme outlined in our original submission, we have purified a set of c-fms 3' UTR binding proteins (Figure 2.). The nuclear protein extract from dexamethasone treated BT20 cells was first purified by cation exchange, then heparin agarose, followed by 3' UTR c-fms RNA affinity column. In each case an aliquot of the flow through and eluted fractions from each column was tested for 3'UTR c-fms RNA binding capacity by RNA gel shift assays. Those fractions which demonstrated functionality by RNA gel shift assay were pooled together for purification by the next step.

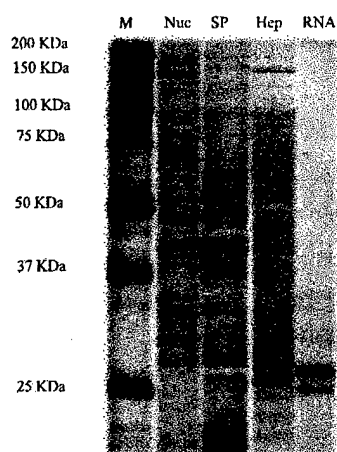


Figure 2. Purification of the c-fms UTR RNA Binding Proteins. Protein samples from the steps of purification were size fractionated on a 12.5% SDS-PAGE gel and stained with silver stain. Lane 1. protein size markers. Lane 2. Nuclear extract fraction from BT20 cells. Lane 3. RNA binding fraction from a HiTrap SP column. Lane 4. active fraction from a HiTrap Heparin column. Lane 5. active fraction from a c-fms 3'UTR RNA affinity column.

Thus, task 1A has been accomplished.

#### **b. Identification and cloning of the RNA binding protein (months 11-12).**

We have submitted the set of purified proteins from the 3' UTR c-fms affinity column shown in Figure 2 for MALDI-TOF MS and for peptide sequencing analysis, and are awaiting the results. Because there appears to be several proteins important to c-fms RNA binding, with some of the proteins being more abundant (eg the 25kDa protein) than others, the amount of purified protein may be insufficient in some cases for sequencing. In that case, we will prepare a larger amount of starting material for repeat purification.

**Task 4.** To search for other proteins stimulated by glucocorticoids in breast cancer cells, which in the future, may prove to contribute to glucocorticoid stimulated regulation of *c-fms* expression

- a. Prepare labeled cDNA probes from RNA from BT20 cells in the presence or absence of dexamethasone, and hybridize these probes to cDNA microarray slides, optimize conditions to improve specificity (months 11-24)
- b. Analysis and interpretation of computer generated data (months 24-27)

RNA from BT20 breast cancer cells in the presence or absence of dexamethasone for 48 hrs was hybridized to an oligo array containing 16,700 known human genes. We were successful with this approach, and could consistently verify differential overexpression by dexamethasone of the following five genes on 4 independent experiments (Table 1). The gene expression profiles of each experiment were analyzed by GenePix Pro 3.0 computer software. The expression profiles of all 4 experiments combined were analyzed using GeneSpring 4.2 computer software for statistical significance.

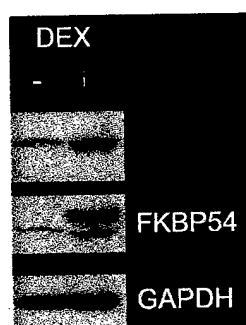
Genes	Expression	Range
FKBP54	5.88	2.79-10.62
GW112	3.74	2.02-5.77
MAO-1	3.60	2.51-5.14
SGK	2.58	1.96-3.48
11 $\beta$ hydroxy steroid DH	2.45	1.94-3.04

Table 1. Five genes were consistently overexpressed in these 4 experiments using a cut-off of 2.0 fold overexpression.

Thus, we have accomplished Tasks 4A and 4B.

**c. Confirm the results by Northern Blot (month 28)**

We decided to confirm our microarray data (when possible) by Western blot analysis.



Measurement of protein expression is a more direct marker of relevance than RNA expression. Overexpression of two proteins by dexamethasone was confirmed (Figure 3). The degree of overexpression by dexamethasone was greater for FKBP54 than for SGK, as suggested by the microarray experiments.

Figure 3. Western Blot analysis of SGK and FKBP54 protein expression in the presence or absence of dexamethasone. Total cellular protein extract from BT20 cells treated with dexamethasone for 48 hrs was analyzed by SDS-PAGE gel and incubated with the respective specific antiserum. After

incubation with secondary antibody complexed with biotinylated horseradish peroxidase, the filters were developed utilizing chemiluminescence. GAPDH served as the control.

Thus, we accomplished all of task 4.

Review of the literature focused on these 5 genes suggested that 3 of them may have a role in hormonally induced actions: FKBP54, an immunophilin related to the progesterone receptor complex is also a natural immunosuppressant; GW112, a recently discovered gene known to be induced by glucocorticoids and differentially expressed in hematopoietic lineages; and SGK (serum- and glucocorticoid-regulated kinase), a serine-threonine kinase known to be induced by glucocorticoids and is also known to be involved in signal transduction pathways.

Thus, we went further to investigate the potential role of SGK in dexamethasone and CSF-1 (growth factor ligand for c-fms) stimulated c-fms related behavior, that of stimulating adhesiveness of BT20 cells to extracellular matrix consisting of type IV collagen and laminin (Table 2). We utilized LY294002 (Ly), a drug known to inhibit the PI3-kinase pathway downstream of c-fms, inhibiting phosphorylation and hence function of SGK.

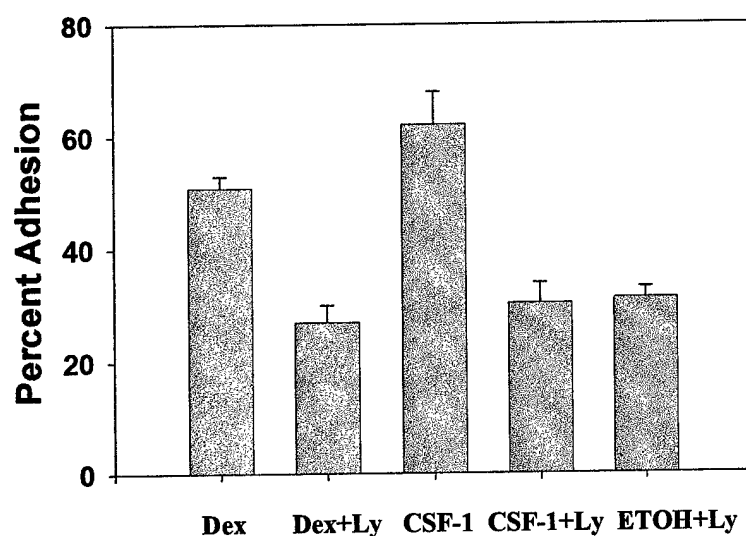


Table 2. LY294002 interferes with stimulation of adhesiveness of BT20 cells to extracellular matrix by dexamethasone and CSF-1. The degree of adhesiveness was measured 2 hrs after seeding treated cells onto a filter coated with type IV collagen and laminin. The filter was stained, and number of adherent cells per well were counted (5 wells per condition), and SEM calculated.

This data suggests that the some of the actions of SGK are downstream of c-fms. Interfering with phosphorylation of sgk by Ly, decreases by 50% both dexamethasone and CSF-1 stimulated adhesiveness of BT20 breast cancer cells. This finding may have implications for potential therapeutic interventions aimed at decreasing c-fms related aggressive breast cancer behavior.

**Task 5. To determine if excess 3' untranslated *c-fms* RNA in breast cancer cells results in down-regulation of glucocorticoid stimulated *c-fms* expression and mRNA stability**

- c. **Clone 3'UTR *c-fms* RNA sequences, containing or lacking the RNA binding protein sequence, into a mammalian expression vector (months 1-12)**
- d. **Stably transfect BT20/ SKBR3 breast cancer cells with these constructs, isolate and characterize the clones (months 6-15)**

The wildtype 3'UTR *c-fms* sequences have been cloned into a mammalian expression vector, and BT20 cells have been stably transfected. The clones are frozen and awaiting characterization.

### **Key research accomplishments**

- By northwestern analysis, we have found that several proteins bind to the 3'UTR of *c-fms* RNA, ranging in size from 25 to 125kDa.
- Some of them are unique to the 69nt RNA fragment within the 3' UTR of *c-fms* RNA identified in the preliminary results, some are not.
- These proteins have been serially purified, and the final fractions retaining functionality as assayed by 3'UTR RNA gel shift assays, have been furthered purified on a 3' UTR *c-fms* RNA affinity column. A set of these purified proteins have been submitted for sequencing analysis.
- By microarray analysis, we have identified 5 genes consistently overexpressed by dexamethasone in BT20 breast cancer cells.
- Sgk, a serine threonine kinase, is one such protein. We have demonstrated that interfering with the function of sgk, significantly decreases dexamethasone and CSF-1 induced adhesiveness of BT20 cells to extracellular matrix.

### **Reportable outcomes**

Tangir J, Bonafe N., & Chambers SK. (March 2003). SGK1, a Potential Regulator of *c-fms* Related Breast Cancer Aggressiveness. Paper presented at: Society for Gynecologic Investigation, 50th Annual Scientific Meeting; Washington, DC. This abstract won the President's Presenter Award, and is included here as an appendix.

Tangir J, Bonafe N, Chambers SK. (January 2003) Glucocorticoid-Induced Genetic Alterations in Breast Cancer Cells. Paper presented at: Society of Gynecologic Oncology, 34th Annual Meeting; New Orleans, LA.

### **Conclusions**

We are well on our way to cloning some of the *c-fms* RNA binding proteins we believe are important to *c-fms* overexpression. Further, we have elucidated some of the downstream components of dexamethasone stimulated *c-fms* behavior. One implication of this finding is that drugs which interfere with sgk function may block *c-fms* related invasive breast cancer behavior. This is important since the sgk gene has not been previously recognized to be important to *c-fms* related phenotypes.

### **References**



None.

**Appendices**

Copy of the abstract presented at the Society for Gynecologic Investigation in March 2003 is appended.

**SGK1, A POTENTIAL REGULATOR OF c-fms RELATED BREAST CANCER AGGRESSIVENESS.** Jacob Tängir,\*<sup>1</sup> Nathalie Bonafe,\*<sup>1</sup>

Setsuko K Chambers.<sup>1</sup> <sup>1</sup>*Dept. of Ob/Gyn. Div. Gynecologic Oncology, Yale University School of Medicine, New Haven, CT.*

**PURPOSE:** Breast cancer cells tend to metastasize early in the course of the disease. This aggressive behavior could be modulated by hormonal mechanisms. Exposure of breast cancer cells in-vitro to glucocorticoids (GC) stimulate their invasiveness, motility and adhesiveness. We have demonstrated that this is largely explained by GC-associated overexpression of the c-fms proto-oncogene, which encodes the receptor for the colony stimulating factor-1 (CSF-1). Binding of CSF-1 to c-fms triggers subsequent intracellular signaling and thereby invasiveness and adhesiveness.

Our objective was to investigate additional GC-associated genetic alterations in breast cancer cells which could contribute to these c-fms related changes in breast cancer behavior.

**METHODS:** Estrogen receptor negative, progesterone receptor negative and GC receptor positive human breast cancer cells (BT20) were exposed to dexamethasone (Dex) for 48 hrs. A microarray technique using a cDNA array representing 16,700 known human genes was used to analyze the gene expression profile of cells exposed to Dex versus non-exposed. Gene expression profiles of each of 4 independent experiments were analyzed using GenePix Pro 3.0 computer software. The expression profiles of all experiments combined were analyzed using GeneSpring 4.2 computer software for statistical significance. Results were confirmed by western blot analysis. Adhesion assays were performed on extracellular matrix containing type IV collagen and laminin.

**RESULTS:** Five genes were found to be consistently differentially overexpressed in the Dex-exposed cells compared to non-exposed after 4 independent microarray experiments: SGK1, FKBP51, GW112, MAO-1 and 11 beta DH. SGK1, a serine-threonine kinase known to be induced by GC, is also known to be involved in intracellular signal transduction pathways. Significant overexpression of SGK after Dex induction was confirmed by western blot analysis. Incubation of the cells with LY294002, a functional inhibitor of SGK action, significantly decreased CSF-1 and Dex induced adhesiveness to the level of control BT20 cells.

**CONCLUSION:** Using microarray technology, a potential regulator of c-fms action has been identified in breast cancer cells. Based on our results, SGK may act as a downstream intracellular regulator of c-fms, particularly of c-fms induced adhesiveness of breast cancer cells after exposure to Dex or CSF-1. This finding may have implications for potential therapeutic interventions aimed at decreasing the aggressiveness of breast cancer cells.